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13. SUPPLEMENTARY NOTES

14. ABSTRACT The objective of this project is to examine the evolutionary consequences of introducing a tetravalent live-attenuated dengue virus vaccine into children in Northern Thailand on naturally occurring endemic wild-type dengue virus. Dengue is the most common arbovirus causing human disease in subtropical and tropical regions of the world and estimated that over 50 million infections occur each year and over 20,000 deaths. In this grant, an interdisciplinary team of university and military investigators have conducted coordinated studies to determine the effect vaccination with a candidate tetravalent vaccine will have on genetic changes on wild-type dengue virus and how these changes will determine risk for severe dengue and serotype-specific dengue virus transmission. Studies are being conducted in Kamphaeng Phet Province, Northern Thailand, to isolate wild-type dengue virus and examine genetic diversity in the population, in hospitalized children with severe dengue illness and cluster investigation of their neighborhoods, and by using sophisticated Global Positioning Systems (GPS) technology of isolated viruses and genetic characterization, spatial and temporal analysis are being performed in detail. This study is a unique opportunity to study the evolutionary consequences of this vaccine on wild-type dengue virus with findings that will have long-term impact on the design of future dengue vaccines and conduct of dengue vaccine efficacy trials.

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Introduction

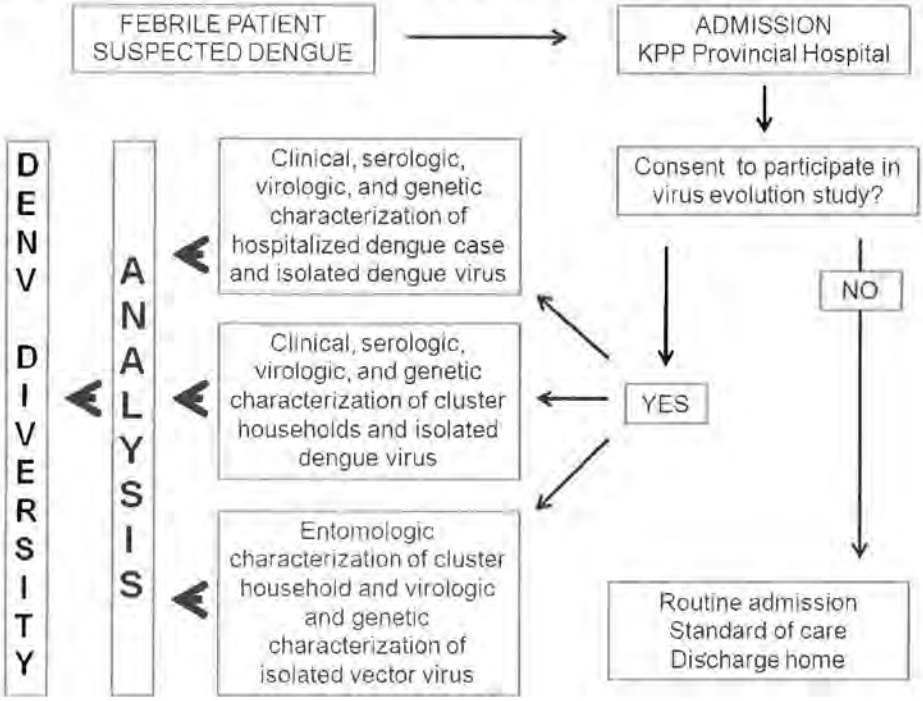
The objective of this proposal is to utilize GPS technology to track dengue disease transmission in Northern Thailand establishing a methodological proof of principal for future investigations. This proposal is part of an NIH supported, Walter Reed Army Institute of Research (WRAIR) approved protocol entitled, "Dengue Virus Circulation, Evolution, Virus-Vector, and Virus-Host Interactions in Kamphaeng Phet Province, Thailand", WRAIR #1526. Dengue is a global health problem and ranks #3 on the DoD infectious diseases threat list. There is no vaccine or specific drug therapy to prevent or treat dengue. Vector control has been unsuccessful at controlling the dengue problem. Personal protective measures require sustained vigilance and may not be possible during a combat or similar operation tempo environment. Increased understanding of vector-virus-host interactions and dengue disease transmission dynamics is required to mount more strategic personal and unit protective measures. Global Positioning System (GPS) technology allows investigators to map, with precision, locations of events of interest; in this case human dengue cases (index), index case contacts that subsequently develop dengue (contact), and mosquito vectors infected with dengue (vector). Advanced molecular techniques allow for complete genetic characterization of viruses (full genome sequencing) isolated from index cases, contacts, and vectors. Tracking events and changes in events clinical/viral/genetic) over time, combined with complex data analysis and geospatial modeling, provide important information about how dengue viruses are transmitted in space in time, how they are evolving, and how viral genotypic evolution impacts disease phenotype (severity). Vector competency assays conducted with viruses and vectors isolated over different geographic regions promote understanding of virus-vector co-evolution and the impact on dengue virus virulence and disease severity. Completing the above before, during, and after execution of a dengue vaccine efficacy trial will provide extremely important information to combatant commanders and vaccine use policy makers about how a dengue vaccine needs to be employed to maintain combat effectiveness during a dengue epidemic or during operations in a dengue endemic environment. The State University of New York-Upstate Medical University (SUNY-UMU) has completed a NIH R01 4-year grant to conduct dengue disease surveillance in Northern Thailand, cluster investigations around the index case, and entomologic investigations and vector competency studies. TATRC funding has allowed GPS mapping of all cases and viral isolates in humans and mosquitoes allowing sophisticated temporal-spatial analyses of dengue disease transmission within the community before, during and after conduct of a dengue vaccine efficacy trial. Sanofi Pasteur had selected the AFRIMS Virology field site in KPP to be a participant in a regional phase 3 dengue vaccine efficacy trial which has been completed. Analysis of both datasets and the influence of a dengue vaccine on wild-type dengue virus evolution is the first of its kind to geospatially track dengue viruses in both humans and mosquitoes and to define dengue virus diversity before, during and after execution of a dengue vaccine trial. The study methodology may be applied to other diseases of interest to the DoD and become standard practice for preventive medicine planning and/or disease outbreak investigations among military units. Deliverables: 1) proof of principal for using off the shelf GPS hardware and software technology in rural environments to conduct detailed and state of the art disease surveillance; 2) detailed information on the dengue serotype-specific virus transmission in the human and vector population in Northern Thailand; 3) geospatial analysis of dengue virus transmission in the human and vector populations; 4) understanding of dengue virus and clinical disease evolution over time and space; and 5) understanding the impact of vaccine introduction on dengue disease and dengue virus evolution over space and time in dengue vaccine recipients and nonrecipients.

Background

Dengue virus has evolved over the last 200 years as four distinct serotypes and an important human pathogen producing severe illness known as dengue hemorrhagic fever (DHF). A dengue vaccine that offers protection against all four dengue serotypes is a high priority of the Department of Defense and based on cost per DALYs saved, highly cost effective. It is not known what effects vaccination will have on the evolution of naturally occurring dengue viruses. Vaccination may create an environment of relative low-transmission of natural dengue virus, within the human host and its vector, thereby increasing stochastic events that will allow new dengue virus genotypes to emerge. These new genotypes could impact virus-vector and virus-host interactions resulting in increased dengue virus

transmission and altered disease phenotype. In this project dengue viruses are collected from hospitalized people with dengue and from febrile individuals residing around the index case and identified through cluster investigations. Integrated and intensive vector surveillance are completed in the area surrounding index cases.

Figure 1 Schematic of Study Activities for Hospital and Cluster Based Investigations.



Dengue vaccine and the effect on DENV evolution

Currently there is no treatment for DHF other than supportive care. A dengue vaccine has been determined to be a high priority and an essential public-health intervention. A number of candidate dengue vaccines are in development. The WRAIR tetravalent live-attenuated DENV vaccine and Sanofi Pasteur chimerivax candidates demonstrated safety and immunogenicity in pre-clinical testing, reduced transmission and replication in mosquitoes, and safety and immunogenicity in phase 1 and small phase 2 human studies [1-5 6 7,8 9-17]. The nature of severe dengue illness as discussed is a complex interaction between the virus and the host-immune response with severe disease being a result of the hostimmune response. Neutralizing antibody to a DENV serotype, the currently recognized correlate of immunogenicity, may not be predictive of protection as demonstrated in studies conducted by our group in Thailand [18]. Current evidence suggests it will be difficult to predict the short or long-term consequences of a dengue vaccine in a population as it is exposed to wild-type DENV [19-21]. From the data presented, it is clear that the DENVs are evolving within specific localities, generating both abundant genotypic and phenotypic diversity as a result of both virus-human and virus-vector interactions, as well as an intrinsically rapid mutational dynamics. Further, stochastic events that result in new genotypes arising during periods of low-transmission, coupled with the possibility of recombination, suggest that DENVs will continue to evolve and produce severe disease. Dengue vaccination offers the opportunity to protect the population from infection and severe disease. Crucially, however, it is not known what effect vaccination will have on the evolution of naturally occurring DENVs. Indeed, based on available data it is possible that several events could occur during vaccination. For example, vaccination may produce an environment of relative low-transmission of natural DENV, thereby increasing the likelihood of stochastic events, in turn facilitating the emergence of new DENV genotypes. Recombination events with vaccine strains may result in attenuated wild-type virus or new genotypes with greater virulence. Dengue vaccination may produce low-titers of enhancing antibody to specific DENV serotypes resulting in emergence of specific serotypes. The vector population requires the human host to be infected. Following a change in the serotype and potential

recombination events, new genotypes may emerge within the vector population posing a risk to humans who are not vaccinated.

Rationale

The AFRIMS Virology field site (KAVRU) is a site for dengue vaccine efficacy studies. It is not known how protective the leading dengue vaccine candidates will be nor the consequences they will have on wild-type circulation and the emergence of genetically unique wild-type DENV. We hypothesize that certain dengue vaccines (live virus or live chimeric) could alter the selection pressure on wild-type DENV by decreasing the transmission pressure on the virus, increasing stochastic events of the virus in the vector population, altering serotype-specific antibody protection or enhancing herd immunity, and potential recombination events with vaccine strains. As a result, it is hypothesized that DENV genetic diversity will increase in and around the vaccinated population allowing the emergence of genetically unique DENVs. Genetically unique DENVs will interact with the host-immune response that has been primed with previously circulating wildtype viruses producing potentially more severe disease.

Military Relevance

Dengue's place in U.S. military history began during World War II (WWII). Soldiers stationed in the Pacific theater introduced dengue viruses throughout Southeast Asia, Japan, and the Pacific Islands. The deployment of non-immune troops to dengue endemic areas with unchecked vector populations resulted in large epidemics of disease. McCoy and Sabin described epidemics among troops in the Northern Territory and Queensland (1942), Espíritu Santo (1943), New Caledonia (1943), New Guinea (1944), and the Philippines (1945). Reports suggest there were over 2 million cases of dengue between 1942 and 1945 in Japan. An extensive dengue outbreak among U.S. forces occurred in 1944 in the Marianas Islands and despite the use of DDT, over 20,000 dengue cases are believed to have occurred on Saipan. Dengue continued to have an adverse impact on the U.S. military during operations in Viet Nam, Somalia, and Haiti. Recent medical literature is documenting significant dengue attack rates among travelers and foreign military personnel deployed to dengue endemic regions. Prevention of dengue through widespread vaccination is an important objective of the U.S. Department of Defense. The U.S. has made significant contributions to dengue research. Ashburn and Craig provided evidence for the viral etiology of the disease making dengue virus the second human viral pathogen identified after the yellow fever virus. Siler, Hall, and Hitchens researched the role of *A. aegypti* as a vector in the transmission of dengue virus. Research performed by Hotta and Kimura and Sabin and Schlesinger during WWII isolated dengue virus types -1 and -2 (DENV-1, -2) and identified the presence of homotypic immunity following infection. In 1956, the dengue epidemic occurring in Manila resulted in the identification and naming of DENV-3 and -4 by Hammon. In the early 1980s, the U.S. Naval Medical Research Unit (NAMRU) No. 2 described dengue outbreaks among U.S. military personnel at Clark Air Base. NAMRU No. 3 in Peru characterizes dengue epidemiology in Central and South America while AFRIMS has been conducting dengue epidemiology and basic science research in Asia for over 40 years. The U.S. military is a leader in dengue vaccine development. Early efforts date back more than 70 years with attempts to prevent virus transmission using infectious human plasma inactivated with formalin. Sabin and Schlesinger undertook the first attempts to immunize using mouse-passaged live-attenuated DENV-1 and -2 viruses. The U.S. Army continued development of these vaccine candidates for the next decade. In 1962, a field efficacy trial in Puerto Rico using the DENV-1 vaccine candidate demonstrated partial protection during an outbreak of predominantly DENV-3. In 1971, the US Army Medical Research Command (USAMRC) launched a program at the WRAIR to develop a live-attenuated dengue vaccine produced in mammalian cell cultures. Meanwhile, Halstead and colleagues demonstrated that dengue viruses could be attenuated in monkeys by passage in primary dog kidney (PDK) cell cultures. A variety of PDK-attenuated vaccine candidates for all four dengue serotypes were manufactured and tested in humans. A down-selection process based on safety and immunogenicity resulted in four vaccine candidates, one for each DENV type, that were combined into a tetravalent vaccine. In 2000, USAMRMC entered into a Cooperative Research and Development Agreement (CRADA) with GlaxoSmithKline Biologics to co-develop PDK passaged live-virus candidates. Phase 1 human studies demonstrated acceptable safety

and immunogenicity in US adults, Thai children and Thai infants. Additional studies evaluated variations in dosing schedule, route and delivery method. Eighteen tetravalent formulations would be tested with two showing promise. Three Phase 2 studies were initiated in the US, Thailand, and Puerto Rico inclusive of over 900 volunteers between the ages of 12 months and 50 years. Adult studies at WRAIR (TDEN-001, N=86) and AFRIMS (TDEN-002, N=120) are complete while the larger study in Puerto Rico (TDEN-003, N=720) continues enrollment (~450 enrolled). In TDEN-001 the frequency of local and general solicited symptoms was similar between the vaccine groups and placebo. Two doses of vaccine were immunogenic, eliciting variable neutralizing antibodies to all DENV types. One dose of vaccine elicited variable CD4+ T-cell responses to all DENV types. An interim analysis of TDEN-002 identified no major safety issues following dose 1 and a significant percentage of volunteers with a baseline naïve or monovalent DENV neutralizing antibody profile being promoted to a tri- or tetravalent antibody profile. An interim analysis of the first 100 pediatric (<21 years) volunteers enrolled in TDEN-003 demonstrated no safety concerns. As seen in previous studies, a single dose of vaccine elicited broad antibody responses in DENV primed volunteers and modest responses in DENV naive volunteers. Final safety and immunogenicity analyses (antibody and CMI) for all studies are pending completion. The WRAIR/GSK dengue vaccine has been safe in over 400 volunteers. Preliminary data supports evaluation of vaccine candidates in a pilot field efficacy trial. After decades of development, the goal of developing a vaccine to protect the Warfighter from dengue appears attainable. As the vaccine development process advances, military investigators and their collaborators are committed to establishing study platforms to understand the near and long term consequences of introducing tetravalent dengue vaccines into endemic populations. Numerous questions and theoretical concerns exist about dengue vaccines and the consequences of their introduction into national immunization programs or, in the case of the military, deployment or entry vaccination schedules.

Body

Hypothesis

We hypothesize that the introduction of a tetravalent dengue vaccine will have important consequences for the evolution of naturally occurring DV (DV) by altering the ecologic pressure on the virus through changes in the virus-host and virus-vector interactions. **The goals of this project are to determine the effect that vaccination with a candidate live-attenuated tetravalent vaccine will have on (1) vaccine related genetic changes that increase risk for DHF in hospitalized children with acute dengue; (2) serotype-specific DV transmission and genetic diversity in the placebo population and breakthrough viremia in the vaccinated population; and (3) the role of mosquito vectors in the emergence and spread of novel DV genotypes.** We propose that dengue vaccination with a live-attenuated tetravalent vaccine will not result in sterile immunity, but produce low-level viremia with subclinical infection from wild-type DV. This will result in selective pressure on specific serotypes to emerge with greater mutational events occurring stochastically as a result of reduced transmission of DV. Mutational events will be reflected in isolated DV in the non-vaccinated population and mosquito vector. We also hypothesize that the effects of selective pressure and cross-reactive antibody protection or enhancement in the vaccinated population will allow the emergence of genetic changes not previously observed in DVs. This will include defective viruses with little pathogenicity as well as the potential for new genetic diversity and recombination events that may result in viruses with greater virulence. An understanding of the genetic events that occur in DV during the field testing of a dengue vaccine will improve our understanding on the longterm consequences of using a dengue vaccine and other candidate DV vaccines on a population, on naturally occurring DVs and its potential effect on unprotected hosts.

Technical Objectives

1. Establish baseline data of wild-type DENV genetic diversity and microevolution in a geographically defined area over time
2. Determine the role of mosquito vectors in the emergence and spread of novel DENV genotypes that may arise from the introduction of a dengue vaccine in a population.

Specific Aims:

1. To conduct a prospective hospitalized-based study of dengue infections and cluster investigations, in order to:
    A. GIS map hospitalized dengue patients and perform cluster investigations of neighborhoods of hospitalized cases to establish data on DENV genetic diversity within a population to determine if diversity varies spatially as a result of local variation in the level of herd immunity and circulating dengue serotypes.
    B. Identify and virologically define virus genetic changes that result in severe hospitalized symptomatic dengue disease.
    C. Characterize virus-host interactions and how DENV genetic diversity impacts these interactions.
2. Evaluate, in a cluster study design, the role of mosquito vectors in the emergence and spread of novel DENV genotypes in order to:
    A. Define the impact of mosquito-virus interactions on the genetic diversity and number of novel DENV genotypes.
    B. Determine whether the emergence and spread of novel DENV genotypes depends on viral adaptation to the local mosquito vector populations.

Methods:

Ethics Statement

The study protocol was approved by the Institutional Review Boards of the Thai Ministry of Public Health (MOPH), Walter Reed Army Institute of Research (WRAIR), and the State University of New York (SUNY), Upstate Medical Center. The IRB's of the University of Massachusetts Medical School (UMMS), University of California, Davis (UCD), and University at Buffalo deferred review to the WRAIR IRB via an inter-agency agreement. All study volunteers and subjects engaged in the informed consent or assent process, as applicable, and documented the same prior to participating in any study activities. In the event the volunteer or subject was unable to participate in the informed consent/assent process, a recognized health care proxy represented them in the process and documented consent. From this point forward, when the authors discuss consent, assent is also implied as applicable.

Prospective Hospital-Based Study of Suspected Dengue Virus Infections and Cluster Investigation- This proposal involves children and adults who present to the hospitals in Kamphaeng Phet with suspected dengue. We will examine the consequences of DENV evolution on dengue disease severity by: (1). GIS mapping of hospitalized dengue patients and cluster investigation in their neighborhoods to determine the level of standing DENV genetic diversity in KPP. To see if diversity varies spatially as a result of local variation in the level of herd immunity and circulating dengue serotypes; (2) identify, quantify and virologically define virus genetic changes that result in severe hospitalized symptomatic dengue disease and determine if these changes are reflected in the larger population; and (3) examine the virus-host and virus-vector interactions by examining the host immune response to variations in the virus.

Monitor Serotype-Specific DENV Transmission and Genetic Diversity in the Vector Population - Our entomological research will be an extension of cluster investigations currently being conducted in Kamphaeng Phet. In this study, we will evaluate the role of mosquito vectors in the emergence and spread of novel DENV genotypes. This is a unique and timely opportunity to study the evolutionary consequences of herd immunity on DENV-vector interactions. Using our existing GIS for the proposed study area, hospitalized symptomatic dengue cases will initiate cluster investigations and collection of infected mosquitoes. Based on prospective longitudinal cluster investigations, we will (1) characterize and compare DENV isolates obtained from mosquitoes collected in areas of index cases and cluster investigations and; (2) monitor the evolution of serotype-specific DENV transmission by conducting lab-based vector competence assays using field-derived mosquito populations and viral isolates from humans with clinical outcomes ranging from severe disease to sub-clinical infection. Mosquito genotyping and virus sequence data will define genetic variation among the mosquitoes and viruses we study. Results of this study will determine whether the evolutionary success of novel DENV genotypes is constrained by genetically diverse mosquito populations.

Study Design

Subject Enrollment: Kamphaeng Phet Province is served primarily by the public health hospital, Kamphaeng Phet Provincial Hospital (KPPPH). Children and adults who are admitted with a diagnosis

of suspected dengue illness will be enrolled in this study. There can be considerable seasonal variation in the annual number of hospitalized dengue cases. Based on a conservative annual clinical attack rate between 1.5-2.0%, PCR positive rates >70% in hospitalized cases and known logistic limitations, investigators anticipate enrolling between 200-800 cases from the hospital each year (seasonal variation) and conducting between 80-150 clusters investigations annually. If enrollment exceeds the upper limit of these projections by >10% investigators will immediately inform the IRB and request an increase in enrollment.

Hospitalized Subjects

Hospital admission evaluation, clinical course and discharge: Patients with an admission diagnosis of acute dengue infection will be contacted by an AFRIMS study nurse and the informed consent and assent, if applicable, process implemented. AFRIMS performs research assays for all suspected dengue cases; all potential volunteers will be known to AFRIMS and captured using this mechanism. Therefore, there will be no advertisements used for this study.

The following information will be recorded from enrolled subjects: demographic information including home address, chief complaint/reason for hospitalization, date of onset of symptoms, symptoms during illness and oral or axillary temperature. Clinical information will be collected from direct interviews and the hospital record. Information will be recorded in the study chart. A blood specimen will be obtained at the time of admission; this specimen is collected by the hospital as part of routine medical care for suspected DENV infections. The clinical course will be charted and based on clinical criteria a classification as DF, DHF and DHF grade, if applicable, assigned. A 2 week (± 5 days) follow-up visit will be completed to obtain a convalescent blood sample. The convalescent visit will occur at the subject's home/school or, in very rare circumstances, the KAVRU field site. Once a parent provides consent for enrollment of their child, the parent does not have to be present for blood collections related to the study. In the hospital and home setting, parents will almost always be present. In the school setting parents will likely not be present. The previous 10 years of dengue studies in KPP were schoolbased studies and blood collection occurred without parents and without incident. In the event a collection needs to occur at KAVRU there will almost always be a parent.

Non-hospitalized Subjects (Cluster Investigations)

Study Population: Cluster investigations of DENV RT-PCR confirmed cases (index case), will be performed throughout the study year. The number of cluster investigations performed per week will be based on the number of index cases and logistical constraints (i.e. index cases residing > 2 hours from KAVRU can not be investigated). Based on past experience, investigators anticipate performing 4-8 cluster investigations per week (total 80-150 clusters per year). Homes of index cases will be GIS mapped and up to approximately 25 susceptible contacts within the household and neighborhood homes will be surveyed for fever or history of fever. Based on the past 5 yrs of cluster studies in KPP, sampling up to 25 contacts was determined to be necessary to obtain a sufficient number of dengue virus susceptible volunteers to consistently isolate viruses, generate useful data for analysis, yet not exceed logistical constraints. Clinical information and acute and convalescent blood samples from consenting subjects will be collected. Mosquito collections will be performed in the household and surrounding neighborhood.

Investigation: Index cases (hospitalized PCR+ subjects) 'triggering' a cluster investigation will be identified between Monday and Thursday of each week throughout the year. Cases will occur during the winter months and these viruses will be a source of diversity during the interepidemic period. Most specimens from index cases will arrive at the field station laboratory by 3pm each day. The DENV RT-PCR result will usually be available by 11AM the following morning. A study nurse will visit the index case and begin the consent process and assent, if applicable, for the performance of a cluster investigation. After the informed consent process is complete and consent has been provided the study nurse will notify an entomological team supervisor to visit the village and begin identifying houses to potentially participate in a cluster investigation (usually within 48 hours of the +PCR result). The exact location of all houses in each participating village will have their location GIS mapped using a Global Positioning System (GPS) unit. Data points will be used to construct a digital map which will enable the team to precisely identify houses located within 100-200 meter radius (the exact radius to be predetermined based on the prevalent average density of homes across all villages) of the index case. The standard cluster radius will be 100m based on the known restricted flight range and the anthropophilic resting and breeding behavior of peri-domestic *Ae. aegypti* populations and

the density of housing in most villages. However, in areas where human dwellings are less concentrated, and there are fewer houses, it may be necessary to extend the sampling unit (cluster) to include more houses between 100-200m from the index case. This approach has been devised based on 5 years of previous cluster investigation experience in the same province and is designed to maximize the scientific output of every investigation. We expect that most clusters will be within a radius of 100 m, but none will exceed a radius of 200 m.Study nurses will visit houses within the 100-200 meter radius starting closest to the index case house and moving outward in a concentric fashion. Each home will be queried for active fever (oral equivalent of >38°C) or a history of fever within the past 7 days. If there is a current fever or history of fever within the household, all household occupants will be offered an opportunity to participate in the cluster investigation. The absence of fever or history of fever will result in no enrollments from that household. A clinical study nurse will complete the informed consent process (Volunteer aged > 18 years old) and assent (Volunteer aged 7-17 years old), if applicable, with potential subjects or the parents or guardians of potential subjects. Those parents (and children) who are unavailable to be consented will be visited that same evening or the following morning; this will be the extent of enrollment attempts. Once up to approximately 25 contacts have been consented, the field teams will be dispatched to the village to begin the investigation. If 25 contacts have been enrolled and there are still residents within the household, investigators will complete enrolling the household (i.e. there may be slightly more than 25 enrollees in a cluster investigation). Clinical information and a blood sample will be obtained from each consenting and assenting, if applicable, subject. DENV RT-PCR will be performed on all acute specimens. Investigators will return to the village in approximately 2 weeks (15 days ±5 days) to acquire a convalescent blood sample and additional clinical information. DENV IgM/IgG ELISA will be performed on all acute and convalescent samples. In the event a subject who is not ill at the time of the acute sample collection becomes ill with fever, investigators will collect a blood sample at the time of the illness and restart the "clock" counting to 15 days ±5 days to acquire a convalescent blood sample. The convalescent visit will occur at the subject's home or, in very rare circumstances, the KAVRU field site. This strategy is designed to maximize acquiring DENV isolates. If a cluster subject becomes ill with a DENV infection and requires hospitalization he or she will NOT become a new index case but followed to completion as a cluster subject. Blood samples will be labeled with a study identifier; the study subject's unique study number, an indicator the sample was collected as part of the hospital or cluster portion of the study, and an indicator that the sample is an acute or convalescent sample (see Annex C). Village leaders will be briefed on the study objectives and design once IRB approval is acquired and before study implementation. Many of the leaders will be familiar with cluster investigations due to recently completed, and similar, activities in the same region. A reasonable effort will always be made to contact the village leader prior to initiating an investigation. If the village leader is available, he or she will be requested to facilitate contact and communication with identified households. AFRIMS has established a precedence for more than 10 years of incorporating the village leadership and residents into the execution of KPP-based dengue studies.

Entomological Investigation: Kamphaeng Phet Province is the site of an AFRIMS' vector field station. Our proposed entomological research will complement the other two aims of this project by addressing the aim to test the following 2 assumptions of our hypothesis (see below) in the context of prospective longitudinal cluster investigations:

a. Genetic diversity of DENVs in mosquito vectors changes as prevailing trends in dengue herd immunity among the local human population changes. Viruses isolated from field-collected mosquitoes will be sequenced and compared phylogenetically across different locations and time-points. The extent and structure of DENV diversity in the mosquito population will be compared with that of the corresponding human population across different geographic locations and across the time course of the study. Based on 5 years of previous work in Kamphaeng Phet we know we will maximize DENV isolations by capturing adult mosquitoes in and around the homes of index cases (see Preliminary Data). Hospitalized symptomatic dengue cases will initiate cluster investigations and collection of infected mosquitoes.

b. The efficiency of DENV transmission by mosquitoes changes as prevailing trends in dengue herd immunity among the local human population changes. We will monitor the evolution of serotype-specific DENV transmission by conducting lab-based vector competence assays with viral isolates from mosquitoes (obtained in *a* above) and humans with clinical outcomes ranging from severe disease to sub-clinical infection. Mosquito genotyping and virus sequence data will define genetic variation among the mosquitoes and viruses we study. Several vector

competence indices (rate of midgut infection, rate of virus dissemination from the midgut to the salivary glands, and virus transmission potential) will be compared experimentally across a matrix of different vector and DENV populations from the same and different locations and the same and different times. The experimental design will allow us to determine whether over time viruses adapt to local mosquitoes populations so that they infect mosquitoes and are transmitted more efficiently.
Research Assays see submitted proposal for details.
Project Milestones: This project is ongoing with the first patients enrolled January 2010 and cluster investigations ongoing. DENV have been isolated from patients, clusters and mosquitoes and currently being sequenced. The dengue peak season will start in May and the protocol is fully engaged to capture all DENV infections per protocol

Results:

Timeline of Accomplishments:

April 2008: Human use protocol and informed consents completed and submitted to the State University of New York, Upstate Medical University (SUNY UMU) for Institutional Review Board approval.
May 27, 2008: SUNY UMU scientific review completed of the protocol and revisions made.
June 16, 2008: SUNY UMU IRB approval of the study protocol. WRAIR IRB submission of the protocol initiated.
July, 2008: NIH award given to SUNY UMU.
August, 2008: Grant subawards to Penn State University and SUNY Buffalo given. Cooperative research and development agreement (CRDA) initiated with the Walter Reed Army Institute of Research and its overseas activity, the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand.
September, 2008: CRADA between SUNY UMU and WRAIR finalized.
November, 2008: CRADA between SUNY UMU and WRAIR signed allowing IRB submission of the protocol to the WRAIR IRB.
January, 2009: Scientific WRAIR IRB review of the protocol completed with changes requested to the protocol language and informed consent. Translation to Thai completed.
February, 2009: WRAIR IRB provision approval given, Thai Ministry of Public Health (MoPH) IRB reconstituted after a 6 month absence allowing submission of the protocol.
March, 2009: Thai MoPH scientific review completed with recommendations made to the informed consent.
April, 2009: Revised protocol resubmitted awaiting final Thai MoPH approval and allowing WRAIR IRB approval and final SUNY IRB validation and study execution.
September, 2009 Protocol and supporting documents version 5.1 dated 22June 2009 were approved by Thai MoPH
November, 2009: Full IRB approvals at WRAIR and given go ahead to execute the study. The first study subjects were enrolled.
Year 2009: Enrollment included only two months of recruitment, November and December. During those two months there were 10 hospitalized index patients enrolled (1 DENV-1; and 3 DENV-2 isolated). Ten cluster investigations were initiated with 108 houses surveyed and GPS mapping completed with 35 cluster contacts enrolled all who did not have dengue infection. There were 91 *Aedes aegypti* mosquitoes collected and one had DENV-1 virus isolated.
Year 2010: From January 2010 to December 2010 there were 89 hospitalized index patients enrolled (15 with DENV-1; 52 with DENV-2; 22 with DENV-3 and 0 with DENV-4). There were 189 houses surveyed for cluster investigation of these index cases and GPS mapped. 403 volunteers were surveyed as part of the cluster investigation with the following viruses isolated: 1 DENV-1; 11 DENV-2; 19 DENV-3; and 0 DENV-4. 3,455 female *Aedes aegypti* mosquitoes were collected within the clusters with 5 DENV-1; 36 DENV-2; 22 DENV-3 and 0 DENV-4 isolated from mosquitoes.
Year 2011: During January to March of 2011 there were 10 hospitalized index patients who were enrolled with DENV isolated, 3 DENV-1; 6 DENV-2 and 1 DENV-3. 16 houses were enrolled and 26 cluster neighbors of the index case. Within the cluster neighbors there were 3 DENV-1 and 2 DENV-3 virus isolated. 47 female *Aedes aegypti* were collected and no viruses were isolated. Successful

completion of one full dengue season. On June 2011 start of the Sanofi Pasteur tetravalent Chimerivax dengue vaccine phase II/III efficacy trial in Kamphaeng Phet.
Year 2012 and 2013: Continuation of the study and data collection with study ending March 2013. See following tables for volunteer enrollment. Serology is being completed for volunteers during year 2013. Full length sequencing of all viruses is near completion with expected completion by September 2013.

Results previously reported in this progress summary (Thomas SJ et al, "Improved Dengue Virus Capture Rate in Humans and Vectors Using Improved Index Case and Contact Surveillance Methods During the 2010 Dengue Season in Kamphaeng Phet Province, Thailand").

Characteristics of the Index Cases

For this analysis, 149 PCR + cases dengue cases were identified at the KPPPH. All met criteria for inclusion as index cases; 93/149 (62.4%) were enrolled. Multiple PCR+ samples in a single day and samples collected outside of the 24-hour window (e.g., Friday afternoon) accounted for the difference between the 149 PCR+ cases meeting inclusion criteria and the 93 ultimately enrolled. All index cases were chosen randomly from those meeting inclusion criteria and all approached for enrollment consented to do so. The minimum age was 2.6 years and the maximum 56 years, with a mean age of 18.7 years (SD 9.4 years). Male to female ratio was 1.1: 1.

Occurrence and Clinical Spectrum of DENV Infections in Index Cases

By definition, each of the 93 index cases was PCR+. Infections with serotypes DENV-1, -2, -3, and -4 were detected in 15 (16%), 56 (60%), 22 (24%), and 0 cases, respectively. Serologic diagnosis indicated acute primary infection in 2 (2%), acute secondary infection in 79 (85%), recent secondary infection in 2 (2%), no serologic diagnosis due to single specimen in 7 (8%); and serology data were unavailable in 3 (3%). The final clinical diagnosis indicated 45 (48%) dengue fever (DF), 24 (26%) dengue hemorrhagic fever grade I (DHFI), 17 (18%) DHFII, 7 (8%) DHFIII, and 0 DHFIV.

Contacts Available for Enrollment

There were a total of 1063 households with contacts eligible for study inclusion; of these 208/1063 (19.6%) contained a person eventually enrolled as a contact. The potential number of contact houses within 200 meters of any index case ranged from 1 to 232 with a mean of 47.7 (SD 42 houses). The range of houses actually enrolled was from 1 to 9 with a mean of 2.2 (SD 1.5 houses). For each index/contact investigation there was a range of potential contacts from 0 to 18 people with a mean of 4.7 (SD 4.0 people) enrolled. See figure 1.

Characteristics of the Contacts

A total of 793 potential contacts were identified, 438/793 (55.2%) were enrolled and serologic data was available for 409/438 (93.4%). The minimum age was 7 months and maximum age 94.2 years; the mean age was 31.4 years (SD 22.3 years). Males made up a slightly lower percentage of contacts than females (46% vs 54%; 1:1.2). People who declined to enroll did so due to fear of donating a blood sample, not being available at the time of enrollment, not willing to participate for unspecified reasons, and having been involved in an index case/contact investigation within the previous 6 months (exclusion criteria).

Occurrence and Clinical Spectrum of DENV Infections in Contact Cases

Of the 438 enrolled contacts 409/438 (93.4%) had a serologic diagnosis available. Evaluable subjects ranged in age from 7 months to 94.2 years with a mean age of 31.4 years (SD 22.3 years). Acute primary DENV infection was diagnosed in 14 (3%), acute secondary infection in 69 (17%), and recent secondary infection in 3 (1%); 322 (79%) had no serologic evidence of infection. There was 1 case (0.2%) with serology consistent with a Japanese encephalitis infection. Among the 86 (21.0%) contacts with a positive dengue serology result, the minimum age was 10 months and maximum 82 years, with a mean of 23.1 (19.6 years). Females represented 55% of cases. Nested PCR results among the 86 revealed DENV-1, -2, -3, and -4 infections in 1 (1%), 14 (16%), 21 (24%), and 0 cases, respectively. PCR was negative in 50 (58%) cases. There were 156/409 (38.1%) subjects with a serologic test result and fever ranging in age from 7 months to 77 years with a mean age of 20.1 (SD 19.5 years). Females accounted for 57%of cases. Acute primary DENV infections were diagnosed in 11 (6%), acute secondary DENV infections in 49 (25%), recent secondary DENV infection in 1 (1%), and no serologic evidence of DENV infection was found in 95 (67%). Therefore, 11/14 (78.6%) acute primary DENV infections, 49/69 (71%) acute secondary DENV infections, and 1/3 (33.3%) recent secondary DENV infections were associated with fever. Fever was associated with 95/322 (29.5%) of contact cases without serologic evidence of DENV infection.

Presentation of symptom data by primary and secondary DENV infection can be found in Figure 2. A significant difference in symptoms was found only in the occurrence of nausea (0% of primary DENV infections versus 35% of secondary DENV infections).

There was a statistically significant difference in the probability of a contact becoming infected based on the DENV type of the corresponding index case. DENV-2 and -3 index case infections carried a much higher probability of infecting a contact (Table 1). Further evaluation of the probability of infection among contacts by age group revealed significant differences within the contacts of index cases with DENV-2 infections.

Spatial Distribution of Contact Households

The spatial distribution of all households and enrolled households demonstrated a statistically significant relationship between the proximity of the household to the index case and the likelihood of reported fever in the household. As distances increased from the index case house, the likelihood of enrollment became lower (see Table 2 and 3).

Also of note and consistent with previous studies, households further from the index case had a lower rate of DENV infection among contacts living within the household. There was a significant decline in the proportion of infected contacts living >120 meters from the index household.

DENV Infection in *Ae. aegypti* Mosquitoes Residing Among Human Dengue Cases

For entomological studies, households were divided into 5 categories (1) all households with potential for enrollment (N=4388), (2) index case households (N=93), (3) non-index households with a PCR+ contact enrolled (N=41), (4) non-index households without a PCR+ contact (N=75), and (5) non-index households without enrolled contacts (N=4229). There were significant differences in the likelihood of collecting mosquitoes and the likelihood that one or more collected mosquitoes was PCR+ for DENV among the sub-groups of households.

A total of 3,565 mosquitoes were collected from 4,438 households (i.e. all households) (Table 4). From all households, 1,288 (29%) had female *Ae. aegypti*; between 0 and 34 female *Ae. aegypti* were collected per household (mean 0.80, SD 2.08). A total of 63 of the 3,565 (1.8%) mosquitoes collected were PCR+ females. A total of 36 of all 4,438 households (0.81%) had a PCR+ female *Ae. aegypti* mosquito. Among index case households (N=93), a mean of 2.49 (SD 4.98) females were collected per house, 51 of 93 households (54.8%) had females, and 23 of all 63 PCR+ females (35.6%) were collected in index case households. Nine of all index case households (9.7%) had a PCR+ female *Ae. aegypti* mosquito. In contact households with PCR+ contacts there was a mean of 2.07 (SD3.66) *Ae. aegypti* females were collected per household, 24 of the 41 (58.5%) had *Ae. aegypti* females, and 1 was PCR+ (1.6% of all PCR+ mosquitoes). In contact homes without a PCR+ contact (N=41), a mean of 2.07 *Ae. aegypti* females (SD 3.66) were collected per home yielding 1 PCR+ mosquito from 1 household (1.6% of all PCR+ mosquitoes). The remainder of non-index case households, those without contacts (N=4,229), had a mean of 0.75 *Ae. aegypti* females collected per household (SD 1.94) and 1,177 (27.8%) households had female *Ae. aegypti*. A total of 36 PCR+ mosquitoes, 57.1% of all PCR+ mosquitoes, were collected from 23 households (0.54% of all households in this category).

There was a significant association between a contact household's distance from the index case household and the finding of PCR+ mosquitoes collected in the contact household. The greatest percentage of positive mosquitoes was in households between 0-40 meters from the index case household; 33 PCR+ of the 561 collected (5.9%). Between 40-80 (7 PCR+ of 545 collected, 1.3%) and 80-120 (13 PCR+ of 733 collected, 1.8%) meters from the index case household rates of PCR+ mosquitoes were roughly equivalent. Contact households 120-160 (7 PCR+ of 749 collected, 0.9%) and 160-200 (3 PCR+ of 981 collected, 0.3%) meters from the index household had very few PCR+ mosquitoes.

Tables and Figures:

Figure 1.

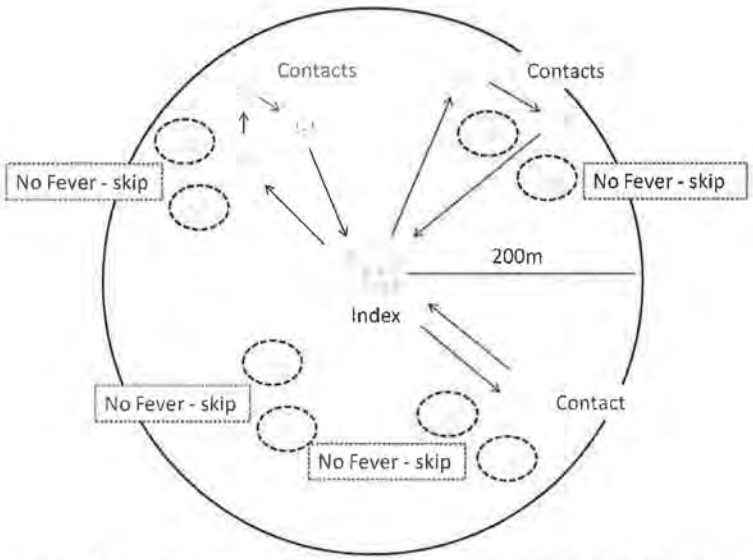


Figure X – Basic design of conducting contact home identification within a 200 meter radius of the index home and identifying those homes with fever or history of fever for possible enrollment.

Figure 2

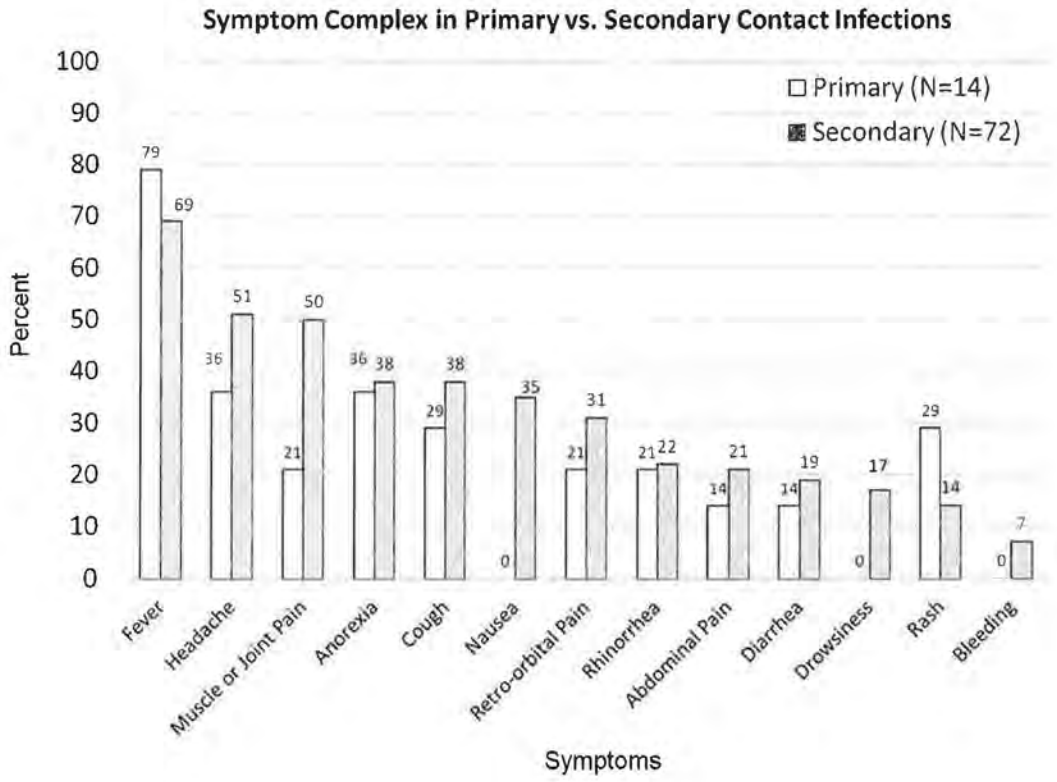


Table 1.
Probability of Dengue Virus Infection in Contacts According to Index Case Infecting DENV type. Numbers in parentheses are the proportions EIA positive. The infecting DENV type is taken from the index case. The differences are significant (Fisher's Exact Test, p-value = 0.003).

Group	DENV-1	DENV-2	DENV-3
EIA Positive	4 (0.09)	44 (0.19)	38 (0.30)
EIA Negative	43	191	88

Table 2.
Spatial Distribution of Households and Enrolled Households – Numbers in parentheses indicate proportions of houses enrolled for each distance category. Differences are significant - Fisher's Exact Test – p-value < 0.001.

	Distance to Index Household				
	0-40	>40-80	>80-120	>120-160	>160-200
Total Households	539	814	971	1051	1063
Enrolled Households	103	53	32	13	7
	(0.191)	(0.065)	(0.033)	(0.012)	(0.007)

Table 3.

Spatial Distribution of Contacts and EIA Positive Contacts – Numbers in parentheses indicate proportions of contacts that are EIA positive for each distance category. Differences are significant - Fisher's Exact Test – p-value < 0.001.

	Distance to Index Household				
	0-40	>40-80	>80-120	>120-160	>160-200
Total Contacts with Serology	228	67	51	38	25
EIA Positive Contacts	56	12	16	0	2
	(0.246)	(0.179)	(0.313)	(0.000)	(0.080)

Table 4. Spatial Distribution of Mosquitoes within Households and PCR Positive Mosquitoes within Households – Numbers in parentheses indicate proportions of mosquitoes that are PCR positive for each distance category. Differences are significant - Fisher's Exact Test – p-value < 0.001.

	Distance to Index Household				
	0-40	>40-80	>80-120	>120-160	>160-200
Total Mosquitoes	561	545	733	745	981
PCR Positive Mosquitoes	33 (0.059)	7 (0.013)	13 (0.018)	7 (0.009)	3 (0.003)

Table one displays the summary information for study years 2009 to 2013. In total there were 323 hospitalized index cases enrolled and 1,246 cluster investigation subjects. More females were enrolled in the cluster-based study as compared to hospitalized index patients. The age distribution as shown with many adults enrolled. There were 457 dengue virus isolations in volunteers of all four dengue serotypes. All viruses were GPS located and currently full length sequencing near completion. Amongst cluster investigation volunteers, there were 235 who were diagnosed with an acute secondary dengue infection. In total 17,283 houses were GPS located during the cluster investigations around an index case. From 2009 to 2013 there were a total of 9,322 female *Aedes aegypti* that were collected; 131 DENV isolated from mosquitoes and GPS located. Full length sequencing of these isolates is being completed.

Table one. Summary Information for study years 2009 to 2013

		Hospital-Base Study	Cluster-Base Study
Number of Enrolled			
Male		166	531
Female		157	715
Age	6months-<7yrs	12	212
	7-13yrs	101	170
	>13-<18yrs	85	85
	≥18yrs	125	779
Summary of result			
DENV-1		75	33
DENV-2		195	67
DENV-3		37	28
DENV-4		16	6
Serologic characterization			
Acute Dengue Infection, need 5-7 days S2 to differentiate			1
Acute Primary Dengue Infection		6	30
Acute Primary Dengue Infection (need HAI to confirm)			1
Acute Secondary Dengue Infection		296	235
Acute Secondary Flavivirus Infection		6	1
JEV Infection			5
Need Follow Up Specimen			1
No Evidence of Recent Flavivirus Infection			909
No Serologic Diagnosis (IgM increasing but non-diagnosti			1
No Serologic Diagnosis (single specimen or less than 5 d		11	40
Recent JEV Infection			1
Recent Secondary Dengue Infection		4	21

Figures one through six demonstrate the results of full-length sequencing to date with completions of DENV-2 and DENV-3. This demonstrates the diversity of DENV in this province yet the spatial conservation of sub-genotypes in neighborhoods that can be mapped and tracked over time. Analysis of this rich dataset is ongoing.

Figure one. Maximum likelihood phylogenetic tree for DENV-2 full genomes.

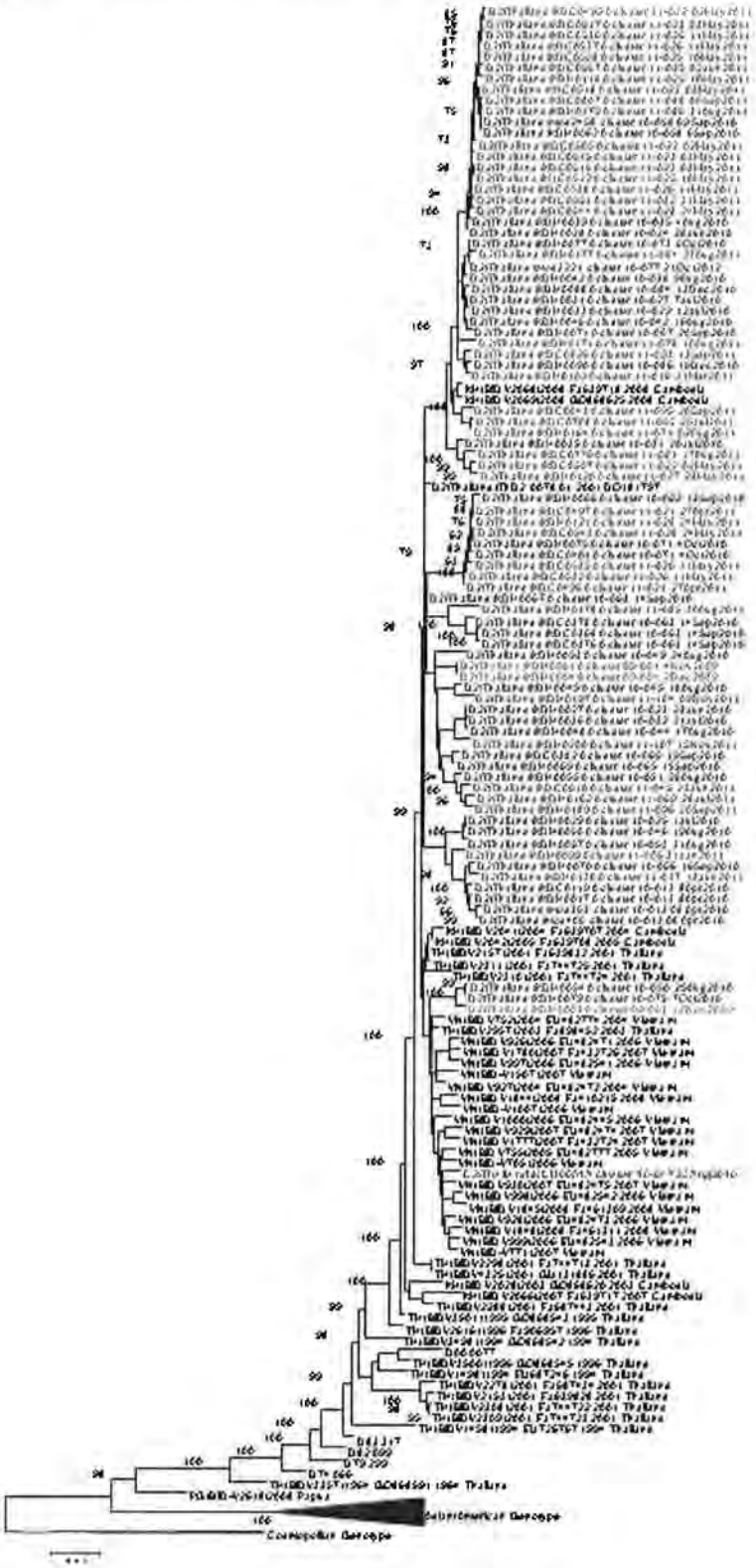


Figure two. Zoomed in maximum likelihood phylogenetic tree for dengue virus serotype 2, genotype I full genomes. Clusters with n>1 samples are highlighted in colors. Samples/clusters in black bold only contained one sample for that cluster.

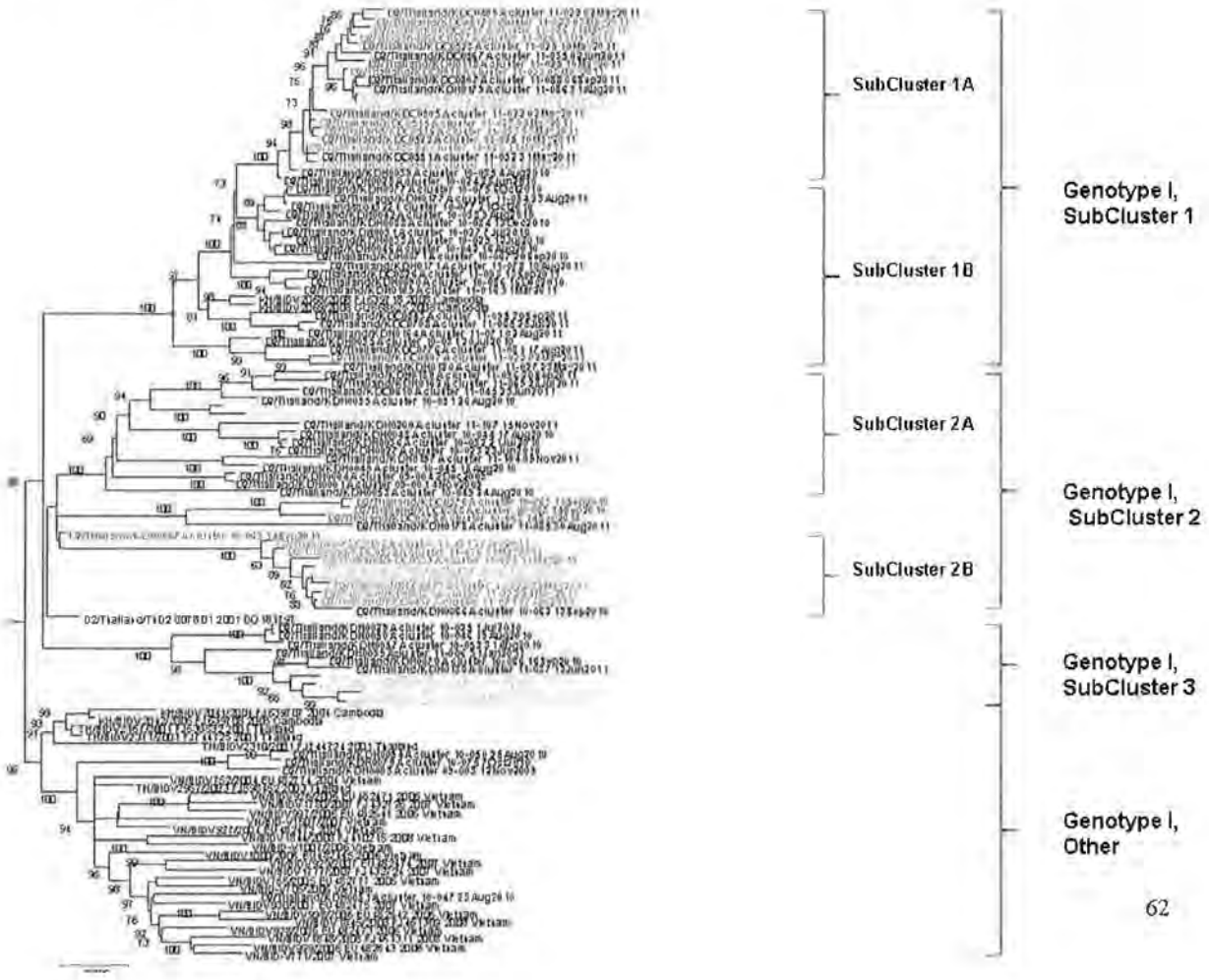


Figure three. DENV-2 cluster locations by GPS and correlation with phylogenetic clusters.

Figure four. Zoomed in maximum likelihood phylogenetic tree for dengue virus serotype 3, genotype II full genomes.

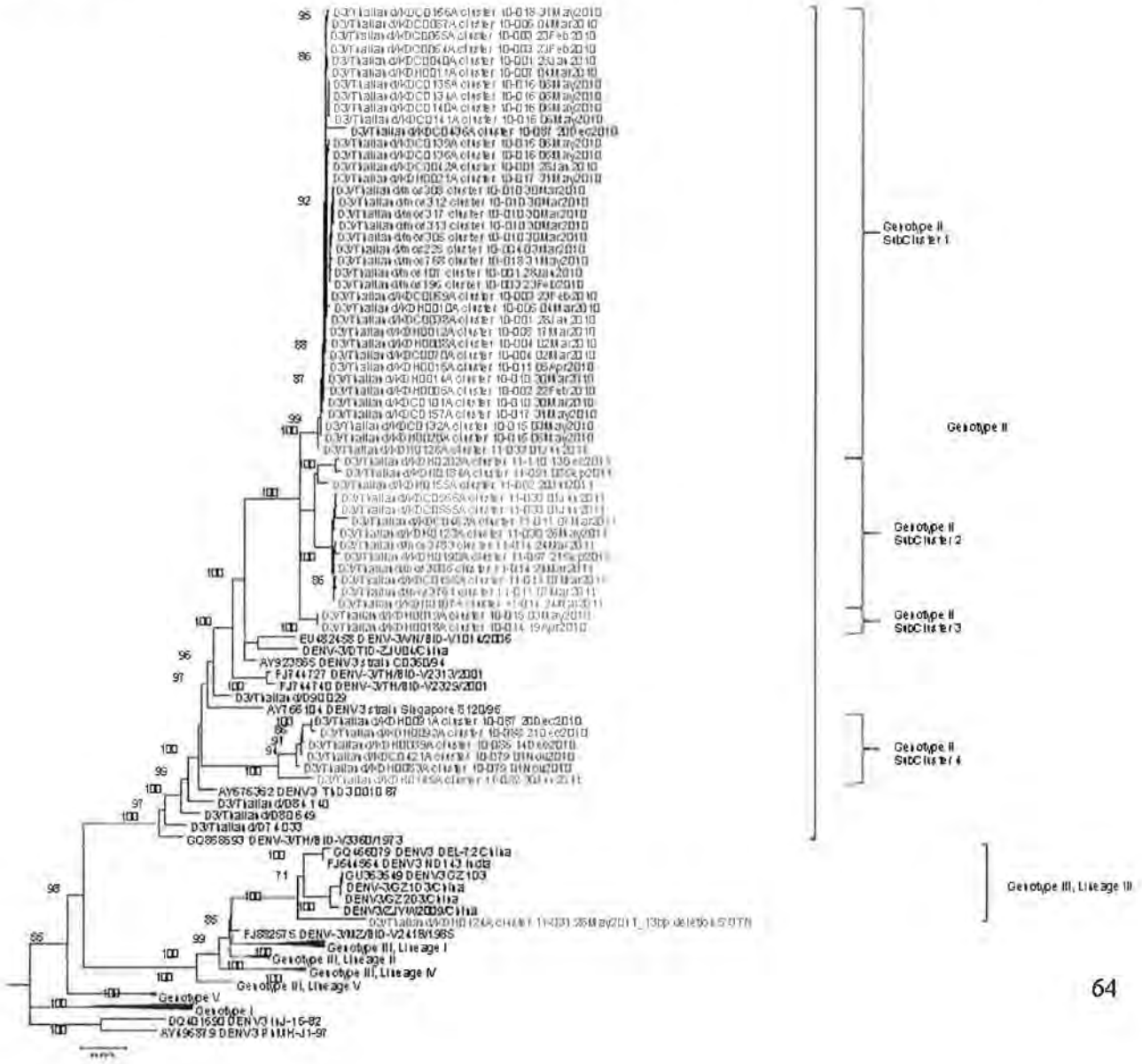


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Figure five. Maximum likelihood phylogenetic tree for dengue virus serotype 3 full genomes.

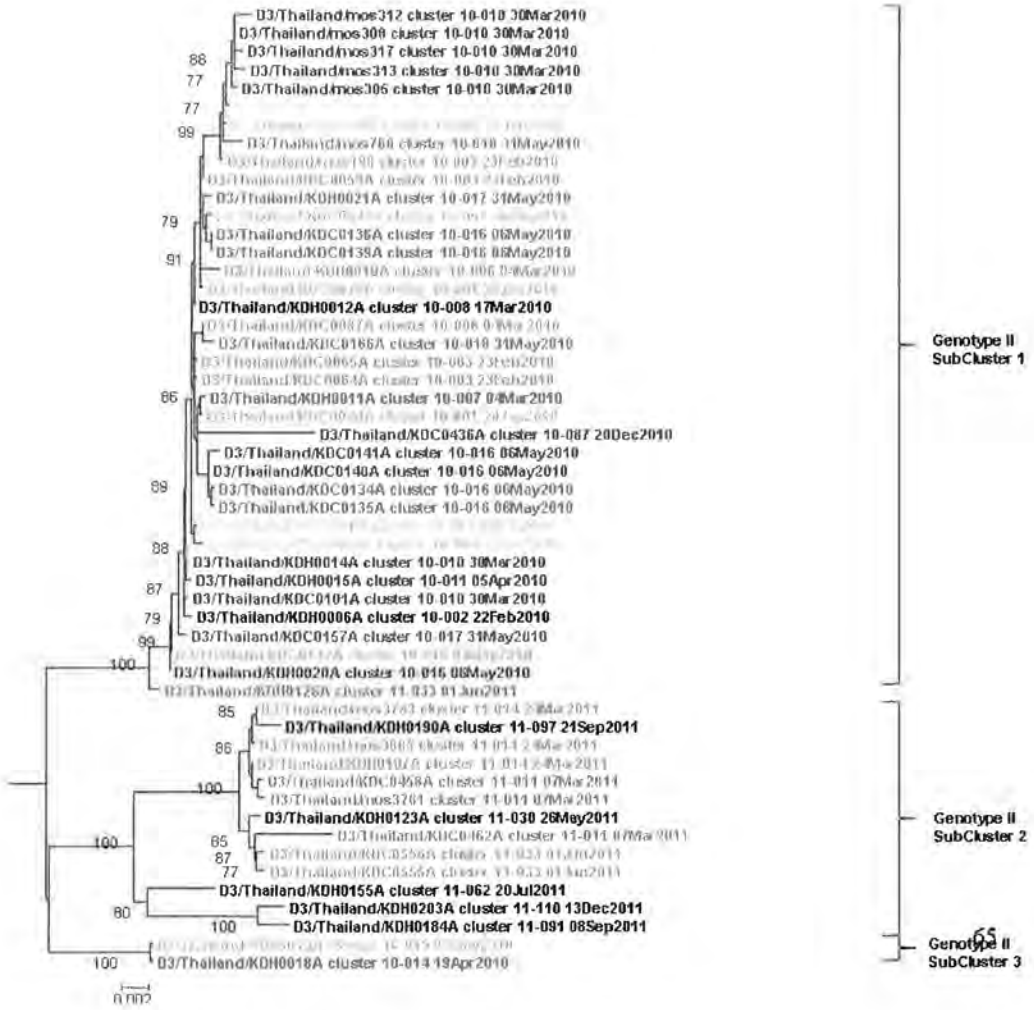


Figure six. DENV-3 GPS mapping and cluster locations.

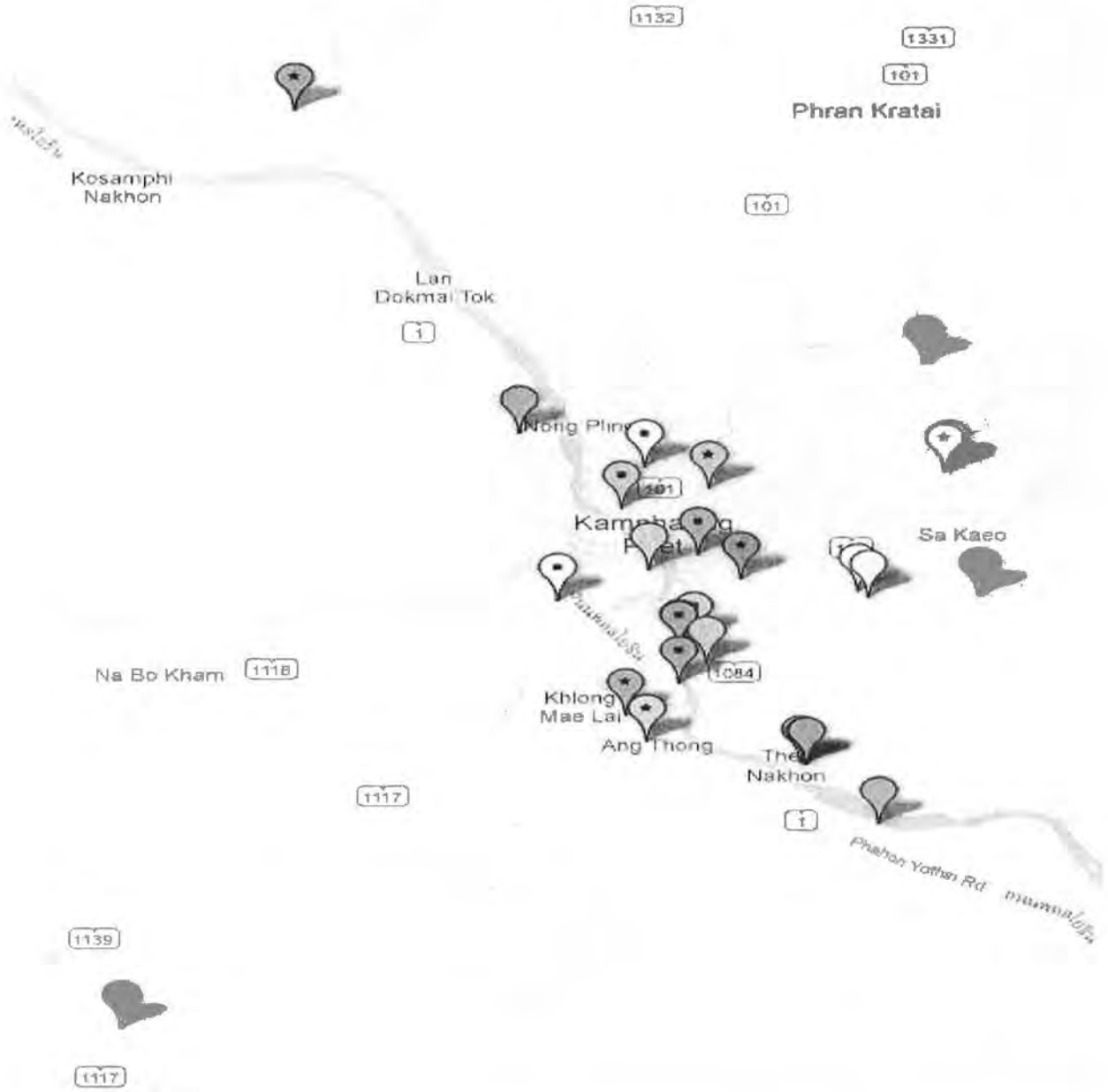
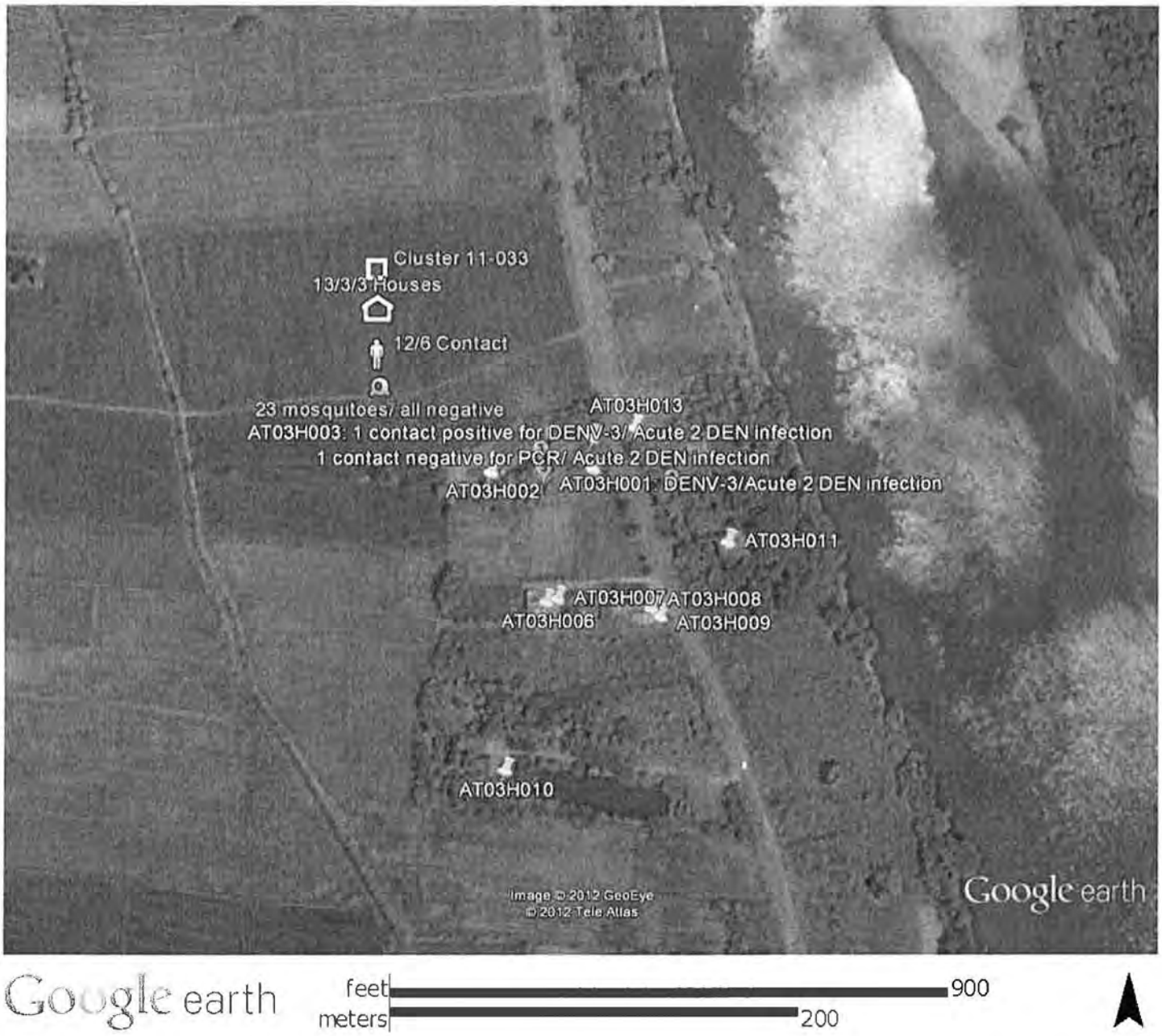


Figure seven. One cluster investigation with GIS mapping using Google Maps.



Significance: The overall objective of this proposal is to examine the evolutionary consequences of introducing a tetravalent live-attenuated dengue virus vaccine into children in Northern Thailand on naturally occurring endemic wild-type dengue virus. Coordinated studies were performed to isolate wild-type dengue virus and examine genetic diversity in the population, in hospitalized children with severe dengue illness and cluster investigation of their neighborhoods, and through integrated intensive vector surveillance, in the vector population. Significant findings to date include: 1) the number of older individuals with symptomatic dengue we are observing is unprecedented from previous dengue studies conducted in this Province and will allow a comparison of the viral isolates by age to see if neutralizing escape mutants are occurring in the population; and 2) overall 8% of cluster contacts and 3% of female *A. aegytpi* had DENV isolated allowing a comparison of sequences with the index cases. There are temporal and spatial differences in this isolation rate with some clusters having high rates of DENV isolated in both contacts and mosquitoes. Spatial analysis is currently ongoing to examine the factors involved in this observation. The field testing of the first dengue vaccine efficacy study offers a unique

opportunity to study the evolutionary consequences of this vaccine on wild-type dengue virus with findings that will have long-term impact on the design of future dengue vaccines and conduct of dengue vaccine efficacy trials.

Plans: This study enrollment is completed and the analysis is ongoing and has already demonstrated unique findings of dengue virus transmission, viral sequencing of isolates from index cases, clusters and mosquito vectors. Analysis is being performed on the micro-evolution of this virus over time and space and the potential effects of a dengue vaccine with incomplete protection.

Project-Generated Resources
This project has generated data and materials (virus isolates and plasma) from children with suspected dengue. These resources can be made available to investigators interested in collaborative research projects; such individuals should contact the Program Director.

Key Research Accomplishments

1. Validated the study design in capturing hospitalized ill index cases and cluster associated dengue infections in a spatially defined area.
2. Demonstrated the association between probability of infection and living close to an index case with dengue infection and virus isolates in mosquitoes.
3. Sequenced all viruses and are currently being analyzed.
4. Demonstrated the value of GPS technology in understanding the spatial and temporal transmission of dengue virus (example in Figure 3 appendix). Two key studies were reported recently from these findings discussed below (papers included in appendix).
5. A protocol has been submitted for IRB approval to do a combined analysis of the R01/TATRC data with data from the Sanofi vaccine trial.

Reportable Outcomes

1. In a publication by Yoon et al, "Fine Scale Spatiotemporal Clustering of Dengue Virus Transmission in Children and Aedes aegypti in Rural Thai Villages" geographic cluster investigations of 100-meter radius were conducted around DENV positive and DENV-negative febrile "index" cases (positive and negative clusters, respectively) from a longitudinal cohort study in rural Thailand [22]. Child contacts and *Ae. aegypti* from cluster houses were assessed for DENV infection. Spatiotemporal, demographic, and entomological parameters were evaluated. In positive clusters, the DENV infection rate among child contacts was 35.3% in index houses, 29.9% in houses within 20 meters, and decreased with distance from the index house to 6.2% in houses 80–100 meters away (p,0.001). Significantly more *Ae. aegypti* were DENV-infectious (i.e., DENV-positive in head/thorax) in positive clusters (23/1755; 1.3%) than negative clusters (1/1548; 0.1%). In positive clusters, 8.2% of mosquitoes were DENV-infectious in index houses, 4.2% in other houses with DENV-infected children, and 0.4% in houses without infected children (p,0.001). The DENV infection rate in contacts was 47.4% in houses with infectious mosquitoes, 28.7% in other houses in the same cluster, and 10.8% in positive clusters without infectious mosquitoes (p,0.001). *Ae. aegypti* pupae and adult females were more numerous only in houses containing infectious mosquitoes. The conclusion was that human and mosquito infections are positively associated at the level of individual houses and neighboring residences. Certain houses with high transmission risk contribute disproportionately to DENV spread to neighboring houses. Small groups of houses with elevated transmission risk are consistent with over-dispersion of transmission (i.e., at a given point in time, people/mosquitoes from a small portion of houses are responsible for the majority of transmission).

2. Aldstadt et al recently published the analysis of GPS data from this grant entitled, "Space-time analysis of hospitalized dengue patients in rural Thailand reveals important temporal intervals in the pattern of dengue virus transmission" [23]. Spatial coordinates of each patient's home were captured using the Global Positioning System. A novel method based on the.Knox test was used to determine the temporal intervals between cases at which spatial clustering occurred. These intervals are

indicative of the length of time between successive illnesses in the chain of dengue virus transmission. The strongest spatial clustering occurred at the 15–17-day interval. There was also significant spatial clustering over short intervals (2–5 days). The highest excess risk was observed within 200 m of a previous hospitalized case and significantly elevated risk persisted within this distance for 32–34 days. The conclusion was that fifteen to seventeen days are the most likely serial interval between successive dengue illnesses.

3. The manuscript entitled, "Improving Dengue Virus Capture Rates in Humans and Vectors in Kamphaeng Phet Province, Thailand, Using an Enhanced Spatiotemporal Surveillance Strategy" by Stephen J. Thomas et al has been accepted for publication by the American Journal of Tropical Medicine and Hygiene.

4. Protocol submitted for combined data analysis with Sanofi Pasteur's vaccine trial. Expected analysis to be completed by December 2014 with manuscript submission by January 2015.

Conclusions

Dengue is an important global and military health problem with no currently licensed vaccine for protection, nor antiviral for treatment. In this novel prospective study of index hospitalized cases of dengue infection and spatial investigation of their surrounding neighborhood using GPS technology, important information is being developed to under the spatial restrictions of dengue transmission. The findings from this study will translate into better interventions to disrupt transmission and guidelines to protect soldiers deployed to dengue endemic areas.

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